VANADATE REGULATION OF CARBOHYDRATE METABOLISM AND MODULATION OF RAT LIVER PYRUVATE KINASE GENE EXPRESSION

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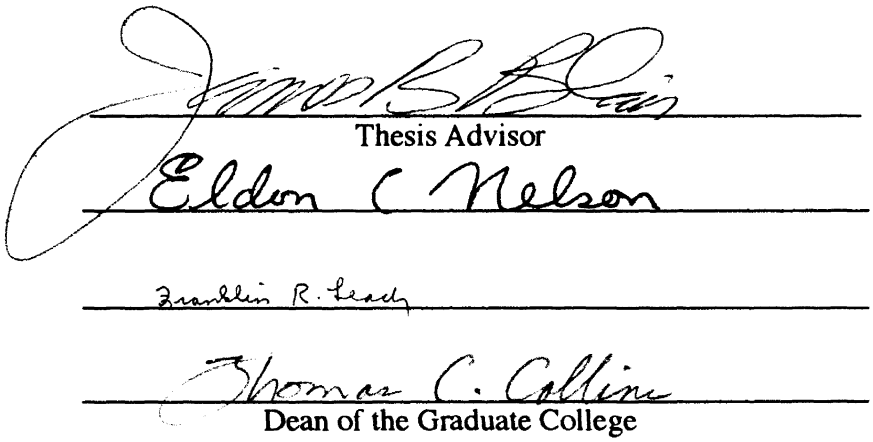

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ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'- triphosphate
ATPase	Adenosine 5'- triphosphatase
cAMP	Adenosine 3', 5'-cyclic monophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBP	Fructose 2, 6- bisphosphate
HEPES	N-[2-Hydroxyethl] piperazine-N'-[2-ethanesulfonic acid]
LDH	Lactate dehydrogenase
LPK	Liver pyruvate kinase
M_R	Molecular weight
NAD	β-Nicotinamide adenine dinucleotide
NADH	β-Nicotinamide adenine dinucleotide (Reduced form)
PEP	Phospho(enol)pyruvate
PK	Pyruvate kinase

CHAPTER I

INTRODUCTION

Vanadate is reported to have insulin-like actions in diabetic rats. The anion lowers blood sugar in diabetic but not normal rats (Ramanadham, *et al.*, 1989; Meyerovitch *et al.*, 1987; and Heyliger *et al.*, 1985). The initial response of blood sugar levels in diabetic rats with 0.8 mg/ml sodium vanadate added to drinking water requires several days, and occurs at a plasma concentration of 15-30 μM (Gil *et al.*, 1988). Several metabolic actions of vanadate have been demonstrated *in vitro* using isolated cell systems or purified enzymes. Metabolic actions of vanadate in isolated hepatocytes and adipocytes from the rat often require very high concentrations of the anion compared to those effective in lowering blood sugar in the diabetic rat (mM versus μM). On the other hand very low concentrations of vanadate are able to inhibit enzyme systems such as the Na^+/ K^+ ATPase and phosphoprotein tyrosyl phosphatase activities. The actions of vanadate in diabetic rats could be related to a specific defect underlying diabetes, and the reversal of the diabetic state by vanadate may take long-term adaptation requiring regulation of gene expression. To address these problems, studies on the uptake of vanadate, and its actions on carbohydrate metabolism and enzymes involved in carbohydrate metabolism in hepatocytes from normal and diabetic rats maintained in short and long-term culture were carried out. In addition, to directly determine if vanadate regulates gene expression of hepatic pyruvate kinase, a chimeric gene construct of the 1000 bp 5'-promoter region of the rat liver pyruvate kinase gene with firefly luciferase was transfected into primary cultures of rat hepatocytes and expression of the chimeric gene product was studied.

CHAPTER II

LITERATURE REVIEW

Although vanadium is a trace element, it is distributed extensively in nature, and is present in almost all living organisms, including Man (Bhagavan, 1983). Vanadium was early recognized for it's ability to inhibit the activity of membrane Na^+ / K^+ - ATPase (Table 1), however now various laboratories document this element is capable of affecting the activity of various intracellular enzyme systems and may modify their physiological functions Skou, (1965) as reported by Bhagavan *et al.*, (1983). Vanadium was discovered by the Swedish scientist N.G. Sefstrom in 1830 and was named after the Scandinavian goddess "Freya Vanadis" (Bhagavan *et al.*, 1983). Bhagavan reported that the first researchers to conduct experiments on vanadium's biological properties were Gamyee and Larmuth, who studied the effects of the element on frog hearts in 1876. Jackson, observed that the pharmacological effects of vanadium were rather similar to those of digitalis (Bhagavan, *et al.*, 1983). In 1977, Cantley and co-workers (Cantley *et al.*, 1977) successfully purified from 'Sigma Grade' ATP a potent inhibitor of (Na^+/K^+)-ATPase which was found identical with sodium orthovanadate (Na_3VO_4).

Vanadium has been recognized as an essential nutritional requirement in higher animals for over two decades. Deficiency results in a general retardation of growth in rats (Bhagavan, *et al.*, 1983) and the supplementation of vanadium in the diet (at subtoxic doses) leads to a variety of metabolic changes including disturbances in sulfur metabolism and cholesterol synthesis.

TABLE 1

METABOLIC PROCESSES AND
ENZYMES AFFECTED BY VANADATE

PROCESS	[EFFECTIVE VANADATE]	REFERENCES
IN VIVO		
Lower Blood Sugar in Diabetic Rats	15-30 μM	Gil *et al*, (1990) Meyerovitch *et al.*, (1987)
Isolated Cell Studies		
Stimulate Glycogen Synthase Stimulate Glucose Transport Stimulate Insulin Receptor	0.2-1.0 mM	Tamura *et al.*, (1984) Green *et al.*, (1986) Mooney *et al.*, (1989)
Inhibit Gene Expression of Liver PEPCK in Hepatoma Cells	0.5-1.0 mM	Bosch *et al.*, (1990)
Raise mRNA Levels for Liver Pyruvate Kinase in Rat Hepatocytes in Culture	25μM	Montserat *et al.*, (1991)
Isolated Enzyme Studies		
Inhibit Na^+/K^+ Stimulated ATP ase	4-500 nM	Courtney *et al.*, (1978)
Inhibit P-Tyrosylphosphatases	2 μM	Swarup *et al.*, (1982)

Vanadium has a wide range of biological and biochemical effects; Bhagavan *et al.*, (1983) reported vanadate's actions on the cardiovascular system (Schmitz *et al.*, 1980), hypertension (Schroeder, 1957), and in manic depression Naylor *et al.*, (1980). However, in relation to this thesis some of the most striking effects of vanadium are related to glucose metabolism, and it's insulin-like effects. Vanadate has been shown to inhibit several enzymes involved in carbohydrate metabolism, and has been reported as far back as 1979 to play a role in modifying blood glucose levels (Simons, 1979; Macara, 1980, and Singh, *et al.*, 1981).

Ramanadham *et al.*, (1990) reported that this latter effect of vanadium was recorded many years ago when Lyonnet *et al* in 1899 observed that a small group of diabetic patients excreted less glucose in their urine when treated with sodium orthovanadate. In addition to being able to stimulate glucose oxidation (Dubyak and Kleinzeller 1980, Clark *et al.*, 1985) , vanadium compounds are known to inhibit lipolysis (Curran, 1954; Snyder and Cornatzer 1958) and to stimulate glycogen synthesis (Tamura *et al.*, 1983). With the recognition of similarities between the physiological response to vanadium and insulin, vanadium compounds have been studied in the treatment of diabetic animals. The first demonstration of the effectiveness of vanadium in the diabetic animal was reported by Heyliger *et al.*, (1985). In their studies, streptozotocin (STZ)-diabetic rats were returned to euglycemic state after 6 weeks of oral vanadate treatment.

Chemistry of Vanadium

Vanadium, a group V element, (mol. wt. 50.9) belongs to the transition series and forms compounds in mainly valence states +3, +4, and +5. It readily changes its oxidation state. In the presence of oxygen, air, oxygenated blood or other oxidizing agents (e.g. diamide) vanadium is almost always in the +5 oxidation state irrespective of the nature of the original compound. Vanadium compounds are in the +4 oxidation state in the presence of reducing agents such as ascorbic acid. Furthermore vanadium can undergo

changes in charge depending on pH. Under physiological conditions at pH 7.4, vanadium in the +5 oxidation state is in the form of an anion vanadate (VO_4^{3-} or it's polyanions), while vanadium in the +4 oxidation state at this pH is predominantly in the cationic form, vanadyl (VO^{2+}). The +3 oxidation state is stable only in acidic solutions (pH less than 2), and in the absence of oxygen. At this pH the +5 oxidation state is prevalent as VO^{2-}, a blue vanadyl cation. Above pH 3, $VO(OH)^-$ begins to appear, followed by the dimer $(VOOH)_2^{2-}$ and then above pH 4.5 by the formation of a precipitate of $VO(OH)_2$.

The +5 oxidation state is rather more complicated since although in acid it occurs as a dioxovanadium cation , VO_2^-, vanadate appears at physiological pH and above as anionic (HVO_4^{2-}) and tends to aggregate to polynuclear complexes. Vanadate also forms complexes with compounds possessing *cis* -glycol functions and with EDTA, and can form polynuclear species with phosphate. The close similarity of vanadate to phosphate accounts for much of its biological interest (Erdman *et al.*, 1984).

History of Diabetes

The name *diabetes mellitus* was introduced by a first century Roman physician, based on the presence of sweetness in urine, the primary symptom of the disease (Koltermann, 1987). The initial concept of the disease was described as wasting of the body with the concomitant passage of large amounts of "sweet urine" leading to death over the course of a few weeks or months. In 1875, Bouchardat pointed out at least two clinical forms of the disease existed in man, one form typically occurring in young individuals who were underweight, and the second form predominant in obese adults. The pancreas became the focus of attention in diabetic research when it was discovered in the late 1800's that pancreatomy in dogs produced the disease. This concept was strengthened by the discovery and isolation of insulin by Banting and Best in 1921 (Koltermann, 1987). Himsworth and Kerr introduced the concept of resistance to the action of insulin in the mid-1930's. The introduction of radioimmunoassay for insulin in

the 1960's soon produced evidence that many adult-onset diabetics had high levels of insulin and, in fact responded to oral glucose loads with insulin levels in excess of those seen in normal individuals.

Classification of *Diabetes mellitus*

The National Diabetes Data Group set forth a system of classification in 1979 that was described to improve the precision with which both physicians and investigators describe diabetic patients. These are as follows;

1. Spontaneous *diabetes mellitus* (90%)of cases.
    a) Type I, insulin dependent diabetes (IDDM).
    b) Type II, non-insulin dependent diabetes (NIDDM).
2) Secondary diabetes.
    a) Pancreatic disease.
    b) Excess counter-regulatory hormones.
    c) Drug-related or drug-induced.
    d) Hyperglycemia associated with complex genetic syndromes.
3. Impaired glucose intolerance.
4. Gestational diabetes: Glucose intolerance that appears during pregnancy.
5. Previous abnormality of glucose intolerance that has apparently returned to normal.

It is Type II (NIDDM) that is of major importance because it is observed in nearly 80% of diagnosed patients (Bergman and Best, 1989). In contrast to type I diabetics, who are usually lean, Type II diabetics are often obese (Koltermann, 1987; Doniach, *et al.*, 1983, and Olefsky *et al.*, 1981). Obesity is such a prominent feature of Type II diabetics that some experts maintain that *diabesity* would be a more appropriate name for the disorder. Although over 90% of Type II diabetics exhibit positive family histories for diabetes, the linkage between genetic mechanisms and pathogenesis of the diabetic state

is not as evident as it is in Type I diabetes. In contrast, insulin deficiency is irreversible in the Type I diabetic (Koltermann, 1987).

Cellular insulin action is initiated by the interaction of the hormone with specific cell surface receptors. As a result of the interaction, a signal or signals are generated that activates the various effector mechanisms required to express insulin effects. The molecular weight of the insulin receptor is approximately 350, 000. The receptor consists of two α-subunits (MW 135, 000 each) and two β-subunits (MW 90, 000). Both subunits of the receptor are glycoproteins, with the glycosidic moieties located on the external surface of the cell. The bulk of the β-subunit is probably located within the plasma membrane, whereas the major portion of the α-subunit is exposed to the extracellular space (Koltermann, 1987). Insulin binds to the α-subunit, and this binding event results in the phosphorylation of the β-subunit. This phosphorylation of insulin receptor initiates a cascade of cellular phosphorylation reactions, resulting in signal transmission to diverse effector mechanisms, which include both membrane processes and multiple enzymatic processes. Therefore, a defect in any step in this cascade of events required for the expression of insulin's cellular effects could theoretically lead to an insulin-resistant state.

The molecular basis of Type II diabetes is still poorly understood despite intensive research. Elevated blood glucose levels in diabetics is the consequence of reduced uptake of glucose by muscle and adipose tissues, and enhanced synthesis of glucose by the liver. In addition, diabetes causes the change in gene expression of enzymes critical in the regulation of carbohydrate metabolism. Also, diabetes is accompanied by enhanced phosphorylation of these regulatory enzymes. One of these enzymes , Type L pyruvate kinase (Blair *et al.*, 1976; James and Blair, 1982; Miyanga *et al.*, 1982; Munnich *et al.*, 1984 and Blair, 1985), is regulated by insulin both through phosphorylation and at the level of gene expression.

The Insulin-Mimetic Effects of Vanadate

Most tissues of higher animals contain intracellular vanadium at a concentration of 0.1-1.0 uM (Simons, 1979). A new interest in vanadium emerged when vanadate was demonstrated to mimic the action of insulin in hexose uptake and glucose metabolism in rat adipocytes (Dubayak, and Kleinzeller, 1980; and Shechter and Karlish, 1980) Some of the other known effects of vanadate are shown in Table 1. The low molecular weight of vanadate and it's analogy to phosphate seems to enable vanadate to permeate plasma membranes and the intestinal wall with relative ease. This can occur by either passive diffusion or by means of an anion carrier. This fact led to Heyliger *et al.*, (1985) attempts at oral treatment of vanadate in diabetic rats. Oral administration of insulin is ineffective.

In their studies high dose streptozotocin-treated rats were used as an animal model because it reflects symptoms of both Type I and Type II diabetes. These rats exhibit low production of insulin and high levels of circulating glucose. The rats were catabolic, insulin-target tissues such as fat, liver, and muscle exhibit insulin resistance in spite of increased levels of insulin binding. Heyliger *et al.*, (1985) used a 4.34 mM Na_3VO_4 solution containing 80 mM NaCl in the drinking water, which had been shown to reduce toxicity. The treatment resulted in the reduction of blood glucose levels and the elimination of depressed cardiac performance. The therapy did not increase the levels of endogenous insulin, therefore insulin target tissues were implicated as the sites of vanadate action (Heyliger *et al.*, 1985).

After Meyerovitch *et al.*, (1987) documented that the conditions utilized by Heyliger and colleagues led to hypoglycemia, Shecter *et al.*, (1988) found that sodium metavanadate ($NaVO_3$) at a concentration of 1.63 mM was the optimal dosage. They found that lower concentrations (0.65 mM) were ineffective. Vanadate solutions were replaced with freshly prepared solutions every 3 days and applied to streptozotocin-rats one week after the induction of diabetes. This resulted in a reduction to normal levels of blood glucose concentration. This effect was reversible. After replacing vanadium

solution with drinking water which contained only 80 mM NaCl, normoglycaemia persisted for another 15 -20 hours after which hyperglycemia recurred.

In addition Shecter *et al.*, (1988) discovered that streptozotocin rats were catabolic in nature (i.e. lost 1g/day) became anabolic by vanadate therapy. The average daily weight gain amounted to + 1.25 g/rat. The rate of hexose uptake *in vivo* was elevated. They showed that vanadate therapy doubled the rate of 3-O-methyl glucose uptake in muscle and liver tissues for control and streptozotocin-rats (Shecter *et al.*, 1988). Thus the normoglycaemia observed in vanadate treated streptozotocin-rats seemed to result from vanadate stimulation of glucose uptake and it's metabolism *in vivo*, in agreement with known actions of vanadate in *in vitro* systems. Schecter *et al.* further documented that 3 wk treatment of ST-rats with 1.63 mM $NaVO_3$ did not raise serum levels of urea, creatinine, and glutamic-oxaloacetic transaminase. Moreover, low vanadate treatment lowered elevated urea levels in streptozotocin-rats to control values. They concluded that several weeks of low vanadate treatment does not result in impairment of renal hepatic functions. They also stated that there might be overlapping site(s) of actions for both vanadate and insulin, and because the insulin receptor is an insulin available tyrosine specific protein kinase, and vanadate is an inhibitor of phosphotyrosine phosphatase, an overlapping site might be a putative endogenous substrate that it's tyrosine phosphorylation and dephosphorylation turns on and off insulin-dependent bioprocesses.

In 1988, Levy *et al.* reported that diabetic animals responded to vanadate treatment in two ways. Streptozotocin rats with their corresponding controls were treated orally with sodium metavanadate. A gradual increase of the vanadate concentration up to 6.55 mM in the drinking water lowered the blood glucose levels of the diabetic animals to normal values without changing the insulin levels. On the other hand, vanadate did not affect the blood glucose levels of the non-diabetic animals, it did however induce lower levels of circulating insulin in these animals. The lowering of the glycemic values of the diabetic animals was closely related to the consumption of vanadate. In addition while the

large majority of the diabetic animals displayed stable normoglycemic values others had fluctuating values. They proposed that the lowering of the blood glucose levels may be due to the stimulation of glucose uptake, glucose stimulation, and glycogen synthesis elicited by the presence of high levels of circulating vanadium. The levels of circulating insulin remained low in the vanadium treated diabetic rats which indicates that the lowering of the glycemic values was probably due to changes in glucose metabolism. Vanadate treated control animals showed low levels of insulin. They proposed that these results seemed to indicate that vanadate treatment, by stimulating glucose uptake, and metabolism, mimic the action of insulin and consequently may reduce its secretion. However, the accumulation of lipid droplets found in the acinar cells of non-treated diabetic animals were reduced after vanadate treatment (Ramanadham *et al.*, 1989). This may be due to a lowering of blood glucose levels and simulation of glucose utilization. Furthermore, they stated that the levels of circulating non-essential fatty acids were also restored with the lowering of the glucose levels by vanadate, and concluded that this was probably related to the reported action of vanadate in stimulating glucose incorporation into lipids in adipocytes and increase in hepatic glycogenesis (Fantus *et al.*, 1989).

In most experiments carried out by researchers investigating the insulin-like effects of vanadium, the element was in the +5 oxidation state as vanadate. However, Ramanadham *et al.*, (1990) reported enhanced *in vivo* sensitivity of vanadyl-treated diabetic rats to insulin. Their study was designed to investigate the concentration-dependent effects of oral vanadyl treatment and possible *in vivo* interaction of vanadyl with insulin in the diabetic animal. While blood glucose levels were affected by all concentrations of vanadyl, 3.4 mM and 4.5 mM produced earlier and greater responses with more consistent decreases. The differential effects on blood glucose were observed though vanadyl intake reached similar levels in all groups. They speculated that the *in vivo* level of vanadyl had to reach a threshold level before the effects started, hence very high concentrations (3.4 mM-4.5 mM) of vanadyl in the drinking water was required.

Furthermore, they noted that because vanadium is rapidly excreted by the kidneys (Hudson, 1964), lower concentrations of vanadyl promotes a more pronounced diuresis and thus a greater elimination of vanadium because it is consumed in larger volumes.

Ramanadham also stated acute administration of submaximal doses of insulin, which was usually only marginally effective in untreated diabetic rats lowered blood glucose levels in vanadyl-treated and vanadyl-withdrawn diabetic animals to near control values within 1 hour. They concluded that treatment of diabetic animals with vanadyl somehow enhanced peripheral tissue sensitivity to insulin, therefore lower endogenous circulating levels of insulin or smaller doses of exogenously administered insulin were required. This was supported by Chaliss *et al.*, (1987) who demonstrated enhanced *in vitro* sensitivity of soleus muscle to insulin, isolated from control animals treated for 2 weeks with vanadate. Their finding also agreed with reports of decreased circulating levels of insulin in control animals while the levels in diabetic rats remained low after chronic treatment with vanadium (Heyliger *et al.*, 1985; Ramanadham *et al.*, 1989). The combination therapy was next attempted in spontaneously diabetic rats. The animals, which absolutely depend on insulin for survival were initially maintained in a control state with daily administrations of insulin (9-13 U/kg) for 14-30 days. Inclusion of vanadyl (0.25 mg/ml) in the drinking water resulted in a gradual decrease in the dosage of insulin (about 4 U/ kg) required to maintain the rats nonglycosuric. Neither this minimal dose of insulin nor vanadyl (0.25 mg/ml) alone was found to be sufficient in glycosuria by themselves. Animals remained healthy and gained weight even though the insulin dose was reduced more than 30%. These results provided further evidence of an enhancement of the *in vivo* effects of insulin in the presence of vanadyl (Ramanadham, 1990).

Vanadate Modulation of Rat Liver Pyruvate Kinase

Pyruvate kinase catalyzes (EC 2.7.1.40) the final committed step of glycolytic metabolism in mammalian tissues.

$$\text{Phosphoenolpyruvate} + \text{MgADP} \xrightarrow[\text{Mg 2+, K+}]{\text{Pyruvate kinase}} \text{Pyruvate} + \text{MgATP}$$

The presence of elevated pyruvate kinase activity in the liver following high carbohydrate meals is consistent with the lipogenic capacity of this tissue in most species (Hopkirk and Bloxham, 1979). However, functional pyruvate kinase activity will result in recycling of phosphoenolpyruvate to pyruvate representing a loss of gluconeogenic capacity to the liver (Katz and Rognstad, 1976). Pyruvate kinase is a unique isozyme, which has kinetic and allosteric properties suited to its position in hepatic carbohydrate metabolism. For example, Tanaka *et al.*, (1967) demonstrated that the enzyme is induced in response to high carbohydrate diet and insulin. In 1976, Blair *et al.*, showed that pyruvate kinase was acutely regulated by glucagon and insulin. These and later observations showed that pyruvate kinase plays an extremely important role in hepatic carbohydrate regulation (Blair, 1981). The present discussion focuses on recent advances concerning the regulatory properties of pyruvate kinase in diabetes with emphasis on the modulation of L-type pyruvate kinase gene expression by vanadate.

Isozymes of Pyruvate Kinase

(Tanaka *et al.*, 1967; Harada *et al.*, 1978;) reported that there are four isoenzymes of pyruvate kinase in mammals, M_1, M_2, L, and R. Type M_2 pyruvate kinase is predominant in the internal organs such as spleen, lung, and kidney. Type M_1 is found in adult skeletal muscle and is a major isozyme in heart and brain. Type R pyruvate kinase is found in erythrocytes. Type L is the major isozyme of adult liver and the only isozyme found in the parenchymal cells of this tissue in adults (van Berkel *et al.*, 1972; Crisp and Pogson, 1972; Garnett *et al.*, 1974). In the rat, type L pyruvate kinase appears in the fetal liver approximately 7 days before birth and the activity rises rapidly until birth (Saheki *et al.*, 1978). Its expression is regulated developmentally, and expression is also regulated

transcriptionally by insulin and glucagon (Noguchi *et al.*, 1985; Thompson and Towle, 1991). The rat liver pyruvate kinase gene is composed of 12 exons and 11 introns with a length of about 9.3 kilobase pairs (Noguchi *et al.*, 1987). The first (exon R) and the second (exon L) exons encode the 5'-terminal sequences specific to the R- and L types respectively, while the remaining downstream exons are common to the two enzymes. Promoter sequences are present in the upstream region of exon R and exon L, respectively indicating that the L- and R-type isozymes of rat pyruvate kinase are produced from the liver pyruvate kinase gene by the use of different promoter sequences. (Yamada *et al.*, 1990).

Studies by Maniatis *et al.*, (1987) indicated that the control of gene expression is achieved through the interactions between *cis*-acting DNA elements and trans-acting proteins that bind to these DNA sequences. *cis*-Acting DNA elements responsible for cell type specific and hormonal regulation of gene expression have been identified (Maniatis *et al.*, 1987; and Roesler *et al.*, 1988). Most of these elements are located in the 5'-flanking region of the gene.

As mentioned earlier, rat L-type pyruvate kinase (L-PK) gene belongs to the category of genes whose expression is induced after feeding a diet rich in carbohydrate. The increase in pyruvate kinase enzyme activity is due a corresponding change in liver pyruvate kinase mRNA levels that result from an increased rate of gene transcription (Vaulont *et al.*, 1986). Induction of liver pyruvate kinase by carbohydrates requires insulin, although both insulin and carbohydrate are required for induction, it appears in the case of the liver pyruvate kinase gene that insulin is not the direct effector, but plays a permissive role by stimulating highly insulin-dependent steps of glycolysis (Mariash *et al.*, 1981). Conversely glucagon can inhibit liver pyruvate kinase gene expression, possibly by reducing glycolysis (Vaulont *et al.*, 1986). When both insulin and glucagon are present the effect of glucagon is dominant (Thompson and Towle, 1991). In order to investigate the induction of liver pyruvate kinase gene expression by carbohydrate,

Thompson and Towle (1991) used a transfection system involving cultured primary hepatocytes. Decaux *et al.*, (1989) demonstrated that primary hepatocytes showed an increase in liver pyruvate kinase mRNA levels when cultured in the presence of high glucose or fructose concentrations, thus providing a useful system for studying carbohydrate regulation of gene expression. Thompson and Towle prepared sequences from the 5'-flanking region of the liver pyruvate kinase gene, which were tested for their ability to direct carbohydrate-stimulated expression of the reporter gene Chloramphenicol acetyl transferase, (CAT) after transfection into primary hepatocytes. They showed that expression of the transfected L-PK (-197) CAT construct correlated with expression of the endogenous liver pyruvate kinase gene under all conditions tested. Thompson and Towle, (1991) eventually localized the carbohydrate response element of the rat liver pyruvate kinase gene to the region between -197 and -96. These 101 base pairs are necessary for glucose-stimulated expression of the pyruvate kinase gene and are sufficient to confer carbohydrate responsiveness to the promoter. This was consistent with the work of Yamada *et al.*, (1990) who identified three positive regulatory elements designated as LPK-I, LPK-II, and LPK-III in the upstream region of the cap site for the L-type isozyme of the liver pyruvate kinase gene. These elements which have synergistic effects on homologous promoters, are necessary for hepatocyte-specific expression of the LPK gene., and are located in the first intron of the liver pyruvate kinase gene, between exon R and exon L. The similarity of the responses of both the endogenous and transfected genes to insulin and glucagon, as well as both glucose and fructose, suggested that alterations in the liver pyruvate kinase promoter activity are primarily responsible for controlling production of liver pyruvate kinase mRNA (Thompson and Towle, 1991).

Miralpeix *et al.*, (1991) showed that vanadate in the presence of glucose raises mRNA levels of L-PK gene. They used the same experimental procedure as described by Decaux *et al.*, (1989). After 5 days of culture in the presence of 10 mM glucose and 10

nM insulin, the cells were cultured for 24 hours in a medium containing lactate but no glucose or insulin. Hepatocytes were then cultured for 24 hours in the presence of 10 μM vanadate with or without glucose plus insulin. When hepatocytes were cultured for 24 hours with vanadate plus lactate in the absence of glucose, after deprivation of glucose and insulin, no accumulation of liver pyruvate kinase mRNA was observed. Glucose alone was insufficient in stimulating the expression of liver pyruvate kinase gene mRNA levels where raised only when glucose and vanadate were present together, as occurred with insulin plus glucose. However the increase of liver pyruvate kinase mRNA levels in the presence of vanadate plus glucose was much less than that observed with insulin plus glucose. The accumulation of liver pyruvate kinase mRNA in the presence of insulin plus glucose *in vitro* was less than 10% of that reached *in vivo* and even lower when insulin was replaced by vanadate (Miralpeix *et al.*, 1991).

The reaccumulation of liver pyruvate kinase mRNA in the presence of glucose plus vanadate or glucose plus insulin was totally blocked by the addition of 50 μM cyclic adenosine monophosphate (cAMP). This inhibitory effect of cAMP was not counteracted by glucose plus vanadate or glucose plus insulin, as had been shown in refed animals (Vaulont *et al.*, 1986). Miralpeix *et al.*, (1991) concluded that vanadate acts similarly to insulin on gene expression, and that the vanadate-glucose-dependent increase of liver pyruvate kinase levels also could depend on the presence of a transcriptional activator derived from carbohydrate metabolism and accumulated in the presence of vanadate plus glucose.

Characterization of Vanadium Uptake

Very little work has been carried out to date on the rate of vanadate uptake and or release of vanadate by cultured cells. Vanadate is taken up by cells through the anion carrier although non-specific uptake may occur (Nechay *et al.*, 1986). In short-term culture systems (1-6 hours), high concentrations of vanadate may be required to obtain

biological responses due to a slow uptake mechanism, thus requiring higher concentrations of the ion in order to achieve sufficient amounts into the cell. Limited uptake may occur since vanadate appears to be taken into cells by an anion carrier, which can be inhibited by phosphate. In living cells, vanadate is reduced to vanadyl by intracellular reducing agents like glutathione (Macara *et al.*, 1980). However, it is often not possible to restrict experiments concerning biochemical or biological aspects of vanadium to one single species, especially when whole cells or organs are involved. For example, in experiments with cultured erythrocytes, vanadium is usually added as vanadate, because only this anion is thought to be able to cross the plasma membrane of such cells (Nechay *et al.*, 1986). Uptake is followed by slow intracellular reduction of vanadate to vanadyl so that both compounds will be present in the cells. Therefore its difficult to ascribe an observed action to a certain vanadium specie (Bode *et al.*, 1990).

CHAPTER III

MATERIALS AND METHODS

Cell Preparation

Isolated hepatocytes were prepared from male Wistar rats (200-300g) obtained from Charles River Laboratories, Wilmington, MA, and fed Purina 5001 Rat Chow, by collagenase (40 mg of Worthington Type II Lot # 48E294F) digestion of a perfused liver, previously described (Bonney *et al.*, 1974; Kleitzen *et al.*, 1976) by Blair *et al.*, (1976) The rats were anesthetized with pentobarbital (50 mg/kg or 0.1 ml./100g body/wt., intraperitoneal). The liver was surgically extracted and hooked up via the hepatic portal vein to a cannula which allowed the perfusion media, filter sterile calcium-free Krebs Ringer Bicarbonate, KRB (200 ml of 0.9% NaCl, 8 ml of 1.15% KCl, 1.5 ml of 2.11% M KH_2PO_4, 1.5 ml of 3.82% $MgSO_4.7H_20$, 42 ml of 1.3% $NaHCO_3$, and 0.1 ml of 400 mg/ml streptomycin sulfate 37°C, 5% CO_2, 95% O_2, pH 7.4, Krebs and Henseleit, 1932 as reported by Berry *et al.*, 1991) to recirculate for approximately 20 minutes, or until the liver was completely digested. After digestion, the liver was removed from the perfusion system and minced in 50 ml of cold perfusion media, and passed through 4 layers of cheesecloth. The resulting suspension of isolated hepatocytes was made up to 100 ml with more perfusion media, gassed with a stream of 5% CO_2, 95% O_2, and centrifuged and resuspended with cold perfusion media three times to remove dead cells and other debris. Cell viability was determined by a lactate dehydrogenase assay which measured the loss of LDH from the cells thus indicating cell rupture. A high percentage of lactate dehydrogenase in the solute signified cell rupture, and any cell preparation which had less than 50 % enzyme recovery in the cells was terminated.

Vanadium Analysis

Preparation of Sodium orthovanadate

Purified grade Na_3VO_4 (Fisher Scientific) was used in all experiments. A O.1 M stock solution was prepared by dissolving solid Na_3VO_4 in 0.1 M HEPES buffer. This gave a colorless solution with a pH of 10. It has been reported that at this pH vanadate is predominantly monomeric as HVO_4^{2-} (Bernier, *et al.*, 1988). The final concentration of vanadate solutions was verified by measurement of absorbance at 260 nm using an extinction coefficient of $3.55 \times 10^3\ M^{-1}\ cm^{-1}$ (Bernier *et al.*, 1988). Immediately before each experiment an aliquot of a freshly prepared stock was diluted into the cell culture medium.

Quantification

The cell samples and 0.5 ml of the media samples were pipetted into 15x75 mm test tubes, and placed in heating blocks at 100°C. They were oxidized by adding 1 part concentrated nitric acid, and 1 part concentrated hydrogen peroxide (H_2O_2). 100 µl of H_2O_2 was added repetitively to all tubes until the last one was completely oxidized, evidenced by a clean tube or the remains of white flaky material at the bottom of the tube. The samples were then ashed in a Muffle Furnace at 300°C for 45 minutes, diluted in 0.5% Nitric acid, Vanadium was then measured by atomic absorption of the element on an Epson Equity III+ Computer controlled Perkin Ilmer 5100 Zeeman Atomic Absorption Spectrophotometer. Ashing and analysis steps of this procedure were kindly performed by Dr. Barbara Stoecker of the Department of Nutritional Sciences. The amount of vanadium was read against standards supplied by Inorganic Ventures, Toms River, NJ., and normalized with the DNA content.

DNA Analysis

DNA content of the cells was quantified using a modified Burton Assay (1956). The cells were scraped off the plates and 200 µl samples (stored in 66% ammonium sulfate, 30 mM 2-mercaptoethanol) used for the assay. To remove endogenous deoxynucleotides / deoxynucleosides, and the ammonium sulfate, the samples were spun down in a microcentrifuge tube at 1000g for 4 minutes. The supernatant was decanted, and the pellet redissolved in 500 µl of 5% trichloroacetic acid (TCA), centrifuged again and the supernatant discarded. The samples were incubated at 70°C for 15 minutes in 600 µl of 10% perchloric acid (PCA), and 500 µl of the resulting supernatant was transferred to a clean tube containing 500 µl of 4 % diphenylamine in acetic acid, and 25 µl of 0.4 % acetaldehyde, mixed well sealed, and incubated at 30°C for 16 hours. Absorbance was determined at 595 nm and calf thymus DNA used as a standard. Each condition was carried out in triplicate.

Metabolic Experiments

Isolated hepatocytes were pipetted in 1ml aliquots into gassed, stoppered scintillation vials, and incubated in a 37°C shaking water bath for 30 minutes. As required the vials contained different concentrations of nutrients and hormones as indicated; sodium orthovanadate, glucagon, and 10 mM dihydroxyacetone. Each experiment was terminated by the addition of 50 µl of 70% perchloric acid. Glucose and lactate levels were analyzed using standardized methods described below.

Glucose Analysis

Fifty µl of concentrated (70%) perchloric acid was added to 1 ml cell samples, allowed to sit overnight, and the transferred into 15 x 75 mm test tubes. Each sample was centrifuged at 1000 r.p.m. for 10 minutes and 50 µl of the solute added to 2 ml of glucostat reagent. Standards were prepared by pipetting in duplicate 0, 10, 25, 50, and

100 µl of a 3.0 mM glucose solution into test tubes containing 2 ml of glucostat reagent. The samples and the standards were vortexed well and the absorbance at 600 nm was read after 1 hour on a Gilford rapid sampling spectrophotometer.

Lactate Analysis

The lactate assay buffer was made by mixing 47 ml of 1.0 M glycine and 38 ml of hydrazine sulfate together and adjusting the pH to 9.2 with NaOH. After the final volume was brought up to 100 ml with water, 78 mg of NAD^+, and 50 µl of 10 mg/ml LDH enzyme (Sigma) was added. 100 µl of the sample were transferred to a tube containing 900 µl of the lactate buffer, and incubated at room temperature for 1 hour. A blank was prepared by adding 100 µl of water to 900 µl of buffer. Absorbance at 340 nm was measured on a Gilford rapid sampling spectrophotometer.

Enzyme Analysis.

Isolated rat hepatocytes from male Wistar Rats (200-300g) were prepared as previously mentioned, and diluted to approximately 800 ml with Swims S-77 (Gibco, BRL), in a sterile 1 liter plastic conical flask, gassed and stored on ice. Ten ml of cells were pipetted on to rat-tail collagen coated 100-mm tissue culture plates. The cells were incubated at 37°C, and 5 % CO_2, 95% 0_2, and allowed to attach for a period of 4 hours. After the initial attachment phase, the media was poured off, and replaced with fresh media containing different concentrations of sodium orthovanadate, low glucose (5.5 mM), or high glucose (25 mM), and 10 nM insulin which was used as a positive control. At this point three plates were removed and used as a "zero time point control". The experiments were terminated after 36 hours by pouring off the media and replacing with 5 ml of cold 66% ammonium sulfate containing 30 mM 2-mercaptoethanol (Foster and Blair, 1978). The plates were stored at 4°C. The cells were scraped from the plates and

centrifuged at 2,500g for 4 minutes. The pellet was resuspended in 500 µl of 66% ammonium sulfate containing 30 mM 2-mercaptoethanol.

Pyruvate Kinase (PK) Assay

50 µl of the cell sample was mixed well with 750 µl of PK Buffer (pH 7.4) in a cuvette. The buffer consisted of 10 ml of 0.4 M Tris-Cl , 5 ml of salt stock (2 M KCl and 1 M $MgSO_4$), 400 µl of NADH (10 mg/ml, Sigma), and 25 µl lactate dehydrogenase (10 mg/ml, Sigma) and 2.5 ml of 15% Triton X-100. The reaction was started by the addition of 25 µl of the substrate mix (100 mM ADP, 100 mM PEP and 10 mM FBP). PK activity was determined spectrophotometrically by the disappearance of NADH at 340 nm and 30°C according to Foster and Blair (1978).

Lactate Dehydrogenase (LDH) Assay

5-10 µl of cell sample was mixed with 1 ml of LDH Buffer (50 ml of 0.4 M Tris-Cl, 10 ml of 10 % Triton X-100, and 40 ml of water, pH 7.4) containing 20 µl of NADH (10 mg/ml). The reaction was started by the addition of 25 µl of sodium pyruvate (40 mg/ml), and change in the absorbance of NADH at 340 nm and 30°C was determined (Foster and Blair, 1978).

Other Reagents

Glucagon

Stock solutions of glucagon were prepared by dissolving 1 mg of glucagon (Lilly Research Laboratories) in 1 ml of water. One µl aliquots of 1 M HCl were added until the glucagon completely dissolved, i.e. when the solution became clear. The final concentration was determined by measuring the absorbance at 278 nm using an extinction coefficient of 8.31 mM^{-1}. Stock solutions were stored at 4°C, and diluted to required

concentrations in media containing BSA which prevents absorption of glucagon on glass surfaces.

Insulin

Stock solutions were achieved by dissolving 1 mg of crystalline porcine zinc insulin (Lilly Research Laboratories) in 1 ml of 1mM HCl, and the concentration determined spectrophotometrically at 276 nm using E 0.1%/1 cm = 1.05. Aliquots of the stock were further diluted as required in media containing bovine serum albumin.

Transient Transfection

Chimeric Gene Constructs

Genelight™ plasmids (Promega) provide a basis for the quantitative analysis of gene expression. They carry the coding region for firefly (*Photinus pyralis*) luciferase which is used to monitor transcriptional activity in transfected cells. Each Genelight plasmid carries the luciferase gene (luc) followed by the SV40 T antigen intron and polyadenylation Poly (A) signals. A second copy of the poly (A) site, located upstream of the luciferase coding region limits background transcription from spurious promoters in the plasmid. The vectors also contain a prokaryotic origin of replication for maintenance in *E. coli* , an ampicillin -resistance gene for selection, and a filamentous phage origin of replication (f1 ori) for single stranded DNA production. Restriction sites for insertion of DNA fragments are located upstream and downstream of the luciferase gene. Two of the upstream sites (XhoI and Bgl II) yield cohesive ends compatible with the downstream sites (Sal I and Bam H I respectively), allowing the interchange of the DNA insert for rapid analysis of positional effects. The Genelight plasmids were pGL2 Basic, pGL2 Promoter, and pGL2 Basic which had the 5'-promoter region of the pyruvate kinase gene inserted in front of the luciferase gene.

The 5'-promoter region of rat liver pyruvate kinase representing approximately 1000 bp upstream from the start site for transcription of the LPK gene was obtained by polymerase chain reaction amplification of genomic DNA by Dr. Raul Espinosa-Nava (personal communications). The regulatory 5'-3' region sequence was obtained from Vaulont *et al.*, (1986) and synthetic oligonucleotides were made as polymerase chain reaction primers.

5' [Hind III]ATGGTATGAT....3' (2166 position) Hind III end = GGAAGCTT

3'TCTGTGTCGTCCATTCG[Bgl II] 5' (3204 position) Bgl II end = TCTACAGGTTC

The promoter was inserted into a plasmid containing the coding region of firefly luciferase (Promega) per instructions of the supplier.

Transfection

Isolated hepatocytes from fed or starved male Wistar Rats (200-350g) were prepared as previously mentioned. The cells were diluted to high density (about 5 ml of packed cells were made up to 600-650 ml in the incubation medium (Table 2). Six ml of cells were pipetted onto rat-tail collagen coated tissue culture plates (60-mm), and incubated for 4 hours . The lipofection method described by Ponder *et al.*, (1991) was used for transfection. After the initial attachment phase, the media was aspirated off using a sterile Pasteur pipette and the cells washed twice with 2 ml of phosphate buffered saline, then 2 ml of transfection media (Table 2) was added. 20 µl of Lipofectin 1 mg/ml, (Gibco, BRL ; Gaithersburg, MD), was mixed well in a polystyrene tube with 1 µg of DNA, which had previously diluted in 20 µl of sterile water. Genelight Basic + Reg 5 plasmid (containing a PK promoter) was used for the experiments. A positive control pGL2 inserted with an SV40 promoter, and a negative control pGL2 Basic Promoter-less plasmid were also used. The DNA/Lipofectin complexes were incubated for 15 minutes at room temperature then 40 µl added dropwise to each plate.

TABLE 2

TRANSFECTION AND INCUBATION MEDIA

Ingredient	Amount per liter	
	Incubation media	Transfection media
Minimum Essential Medium (Sigma)	750 ml	750 ml
Waymouth (Gibco)	250 ml	250 ml
Bovine Serum Albumin	2g	
Alanine	40 mg	40 mg
Serine	52.6 mg	52.6 mg
Penicillin G	60 mg	
Streptomycin sulfate	100 mg	
Fungizone	0.25 mg	
Insulin	1 mg	
Glutamine	292 mg	
Human Growth Hormone (2.4 IU/mg)	250 µl	25 µl
Dexamethasone		0.78 mg
3, 5, 3' - triiodo-L-thyronine, T_3 (1 mg/ml)		673 µl

After 16 hours in a 5 % CO_2 incubator maintained at 37°C, the medium was aspirated and replaced with fresh transfection media supplemented with effectors as specified. The cells were maintained in culture for another 12-24 hr. Experiments were terminated by aspirating off the media, and freezing plates at -20°C.

Luciferase Assay

A reporter system in which firefly luciferase activity was measured to determine the level of expression of transfected PK. The cells are lysed with a Lysis Reagent (Promega) to release the reporter protein luciferase. Both ATP and the substrate luciferin were added to the lysate in a luminometer. The enzyme catalyzed a rapid, ATP-dependent oxidation of the substrate, which then emits light. Total light output was measured and is proportional to the amount of luciferase present over a wide range of enzyme concentrations (Brasier *et al.*, 1989 and Williams *et al.*, 1989).

The Luciferase Substrate (Promega) was dissolved in 10 ml of Luciferase Assay Buffer (Promega). Dilute 1 volume of the cell culture lysis reagent, 5X (Promega) with 4 volumes of water to produce a 1X stock. The cells were thawed, incubated at room temperature for 15 minutes in 250 µl of 1x cell culture lysis reagent, scraped into microcentrifuge tubes, and centrifuged for 5 seconds to pellet debris. Luminescence was read on a model 2010 A Lumac luminometer, using 40 µl of the extract. The assay was linear over a wide range beyond that found in the liver extracts as demonstrated using purified luciferase supplied by Dr. Franklin Leach.

CHAPTER IV

RESULTS AND DISCUSSION

Vanadate Accumulation in Rat Hepatocytes

Vanadium accumulation in isolated rat hepatocytes was studied to estimate the concentration in the cells after long and short term exposure to the anion. This was carried out to see if the levels of vanadium accumulated by rat liver hepatocytes correlated with the concentrations necessary for its actions on carbohydrate metabolism, and gene expression. In order to do this several assumptions were made : 1) Each plate or vial contained the same amount of cells, 2) The carry over of vanadate at the termination of each experiment is negligible, and 3) The vanadium measured in the cell pellet was mainly intracellular. Due to the large cell/media volume ratio, there were also difficulties in measuring the cell volumes accurately. Short and long term exposures were initiated because these time frames covered the time needed for vanadium to exert it's effects on glucose metabolism, change in enzyme activity, and gene expression.

Short-term Experiments

Isolated hepatocytes were incubated in 50 ml of incubation media containing different concentrations of sodium orthovanadate (Na_3VO_4). The flasks were gassed with 5% CO_2, 95% O_2 every 30 minutes and kept stoppered while rotating in shaking water bath (at 37°C) for the duration of the experiment. At 60, 120, and 240 minute time intervals 1ml aliquots were removed, and centrifuged at 10, 000g for about 10 sec. 0.5 ml

of the supernatant and the whole pellet were kept for vanadium analysis. This was carried out in triplicate for each concentration of vanadate at that particular time point. A similar experiment was done using 1mM vanadate, and shorter time points. The volume of cells in these experiments was determined before addition of the incubation media , and was assumed to remain constant over the incubation period.

Table 3 shows that the uptake of sodium orthovanadate in short-term cultured hepatocytes occurred rapidly. Extracellular vanadium concentration over these periods of time also showed a significant decrease. Vanadate rapidly accumulated to approximately the same concentration as the extracellular media, and at high (1 mM) concentrations this occurred as early as 15 minutes or earlier. All the vanadium was accounted for by adding up the amount of vanadium in the cells and the media at the end of each experiment and seeing if it was essentially the same as the total vanadium in the original media.

Long-term Experiments

These experiments were carried out over a 12-24 hour time period. Isolated hepatocytes were plated on rat-tail collagen coated 100-mm Corning tissue culture plates. They were incubated for an initial attachment period of 4 hours in a 5% CO_2, 95% O_2 water jacketed incubator set at 37°C. After this the media was decanted off and replaced with fresh media containing different concentrations of vanadium. The experiment was stopped at 12 and 24 hours by pouring off the media and washing the cells with cold isotonic saline. The saline was decanted and the plates and media were frozen at -20°C for subsequent analysis. A control was achieved by exposing the cells to vanadate and immediately stopping the experiment as mentioned above. The cells were then thawed and scraped from the plates with the aid of a 'rubber policeman', and transferred into 15x75 mm test tubes. Cells cultured for longer periods of time in 10 uM vanadate showed a significant increase in intracellular vanadate (Table 4).

TABLE 3

SHORT-TERM UPTAKE OF VANADIUM BY FRESH ISOLATED RAT HEPATOCYTES

Experiment I

Condition	Media		Cells		Total
	nmoles	[Vanadate] μM	nmoles	[Vanadate] μM	V nmoles
Original media	23.5±1.8	46.3±3	NM	NM	23
No vanadate	0.0±0*	0.0±0*	0.0±0*	0.0±0*	0
Zero min.	23.6±0.6	47.2±1.2^{z}	0.0±0*	0.0±0*	23
60 min.	16.8±0.5	33.5±0.9^{x}	1.5±0.2	65.3±7.8^{c}	19
120 min.	19.5±0.3	38.9±0.6^{y}	1.2±0.01	48.3±0.4^{b}	21
180 min.	22.0±0.5	43.9±0.9^{y}	0.9±0.1	39.9±2.1^{a}	23

Experiment II

Time (min.)	Media		Cells		Total
	μmoles	[Vanadate] mM	μmoles	[Vanadate] mM	V μmoles
Original	0.35±0.03	0.70±0.05	NM	NM	0.35
No Vanadate	0.0±0*	0.0±0*	0.0±0*	0.0±0*	0
zero	0.19±0.05	0.37±0.10^{x}	0.16±0.01	1.3±0.1^{a}	0.35
15	0.25±0.05	0.50±0.02^{y}	0.16±0.01	1.3±0.1^{a}	0.41
30	0.24±0.01	0.47±0.09^{z}	0.16±0.01	1.3±0.1^{a}	0.40
45	0.28±0.02	0.55±0.03^{z}	0.17±0.04	1.4+0.3^{a}	0.45
60	0.24±0.05	0.47±0.10^{z}	0.17±0.02	1.4±0.2^{a}	0.41

*Vanadium was measured but was not detectable
NM= Not measured
The amount of sodium orthovanadate taken up by cells exposed to different concentrations of the anion is presented as the mean ±S.E.M. of three determinations for each condition Numbers with the same letter are not significantly different. $p < 0.01$.

TABLE 4

VANADIUM UPTAKE BY LONG-TERM PRIMARY CULTURE RAT LIVER HEPATOCYTES

Condition	Media	Cells		
	[Vanadate] μM	mg DNA per plate	nmoles V per plate	Estimated [Vanadate] μM
		Experiment I		
No Vanadate				
0 h	ND*	0.12±0.01	ND*	ND*
12 h	0.03±0.01	0.12±0.01	ND*	ND*
24 h	0.06±0.02	0.11±0.02	ND*	ND*
0 h	9.1±0.1	0.10±0.01	0.5±0.1	5±1[a]
12 h	7.7±0.2	0.12±0.01	1.05±0.01	8±1[b]
24 h	6.8±0.03	0.12±0.02	1.99±0.10	16± 5[c]
0 h	167±3	0.11±0.03	4.9±0.5	45±7[d]
12 h	161±2	0.10±0.02	7.4±0.2	68± 7[c]
24 h	147±17	0.11±0.01	10.7±0.1	90±5[f]
		Experiment II		
No Vanadate	ND*	ND*	ND*	ND*
1μM Vanadate				
0 h	1.5±0.1	0.12±0.02	ND*	ND*
18h	1.6±0.2	0.09±0.01	0.12±0.03	2±0.1[a]
36h	1.7±0.5	0.09±0.01	0.13±0.03	2±0.4[a]
10 μM Vanadate				
0h	9.9±1.7	0.10±0.03	ND*	ND*
18h	7.5±0.4	0.10±0.01	0.4±0.1	5±2[b]
36h	6.5±0.6	0.09±0.02	0.8±0.2	11±3[c]

* ND= Vanadium was measured but not detectable

Vanadate accumulation in rat liver hepatocytes exposed to different concentrations of vanadate is presented as the mean ± S.E.M. n=3. Numbers with the same letter are not significantly different. $p < 0.01$

At 12 hours the concentration had equilibrated with the media. At higher concentrations (167 μM) the cellular content still did not attain high amounts, but reached levels close to the media concentration.

Regulation of Carbohydrate Metabolism by Vanadate

Isolated hepatocytes from fed and fasted male Wistar rats were pipetted in 1ml aliquots into gassed, stoppered scintillation vials, and incubated in a 37°C shaking water bath for 30 minutes. As required the vials contained different concentrations of sodium orthovanadate, glucagon, and 10 μM dihydroxyacetone.

Experiments were performed to analyze:

1) Glucose and lactate production from endogenous glycogen in fed rats, (Table 5) in response to increasing concentrations of glucagon, without vanadate, and in the presence of 1mM vanadate. Vanadate resulted in a shift in the glucagon dose response curve. The magnitude of the response of the cells to glucagon was the major effect, and 1mM vanadate lowered glucose output from endogenous glycogen in all concentrations of glucagon, and in the control. Vanadate was also capable of overcoming the inhibition of lactate formation by glucagon in fed animals. Table 5 shows that the effect of vanadate became significant at around a glucagon concentration of 1 μM.

2) Glucose and lactate production from 10 mM dihydroxyacetone in isolated hepatocytes treated with different vanadate concentrations, with and without glucagon. from 18 hour fasted rats. In these types of experiments the rats were fasted because its been demonstrated that the levels of glycogen in fasted rats are extremely low, therefore any glucose produced by the end of the 30 minute experiment came from gluconeogenesis via dihydroxyacetone, an added substrate. This enabled me to follow the glycolytic / gluconeogenic pathway in both directions, the formation of glucose and the opposite way, the formation of lactate. A vanadate dose curve (Figure 1) shows that increasing the concentration of the anion reduced glucose production in control cells, but

the effect was more pronounced in hepatocytes given 1 μM glucagon. The anion was able to raise lactate production to control levels in rats incubated in 1 μM glucagon (Figure 2), until the vanadate concentration reached 1mM, when lactate production had fallen drastically.

Table 6 shows that 100 μM vanadate was able to significantly reduce glucose production in both control and 1 μM glucagon treated cells. As in the fed animals, glucagon did not increase the cells sensitivity to vanadate, but just increased the magnitude of response. Cells exposed to glucagon produced more glucose than the control cells exposed to the same concentration of vanadate. Lactate production was significantly increased by 50 μM vanadate in both control and glucagon treated cells.

TABLE 5

EFFECT OF 1 mM VANADATE ON GLUCOSE AND LACTATE PRODUCTION IN FED RATS IN RESPONSE TO INCREASING CONCENTRATIONS OF GLUCAGON

[Glucagon] M	Glucose Production µmoles / ml packed cells		Lactate Production µmoles /ml packed cells	
	No Vanadate	1mM Vanadate	No Vanadate	1 mM Vanadate
0	11.64±0.64[a]	3.43±0.47[f]	11.86±0.29[i]	12.66±0.37[i]
10^{-10}	15.43 ±1.08[b]	3.45±0.91[f]	11.59±0.41[ij]	11.59±0.22[j]
$3x10^{-10}$	16.75±0.50[c]	4.23±0.43[fg]	11.18±0.25[j]	11.39±0.21[jk]
10^{-9}	17.83±0.41[d]	4.28±0.34[fg]	11.07±0.10[j]	10.99±2.12[k]
10^{-8}	17.92±0.20[d]	5.30±0.99[h]	10.40±0.15[k]	10.59±1.08[kl]
10^{-6}	21.17±4.25[e]	5.77±0.65[h]	8.28±0.65[l]	10.18±0.79[l]

Glucose and lactate production in isolated hepatocytes incubated at 37°C for 30 minutes, from a fed rat .
Results represent the mean ±S.E.M. of triplicate conditions in at least three experiments
Numbers with the same letters are not significantly different. $p < 0.01$

Figure 1. The Effect of Vanadate on Glucose Synthesis from 10 mM Dihydroxyacetone. Glucose production in fresh hepatocytes over a 30 minute incubation period at 37°C was determined as described in the Material and Methods is plotted versus increasing concentrations of sodium orthovanadate. The cells were incubated for 30 minutes at 37 °C. Each point represents the mean ± S.E.M. of triplicate conditions. Similar results were obtained in at least two separate experiments.

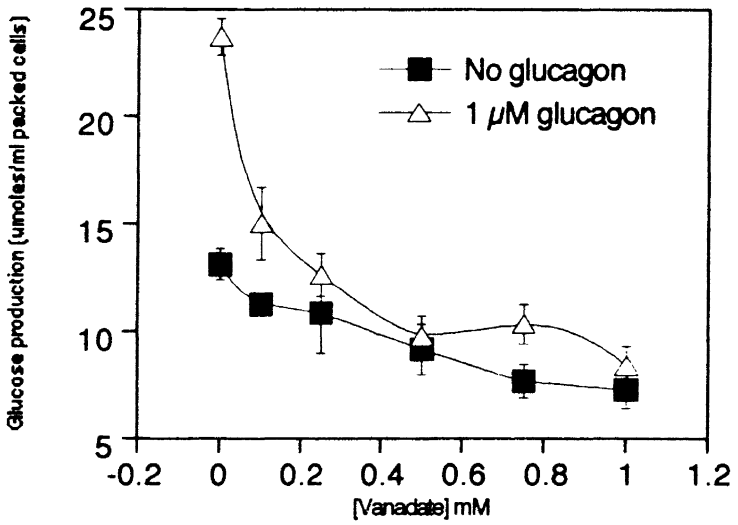
Glucose production (umoles/ml packed cells)
25
20
15
10
5
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
[Vanadate] mM
No glucagon
1 μM glucagon

Figure 2. The Effect of Vanadate on Lactate Production From 10 mM Dihydroxyacetone
Lactate production in fresh hepatocytes over a 30 minute incubation period at 37°C was determined as described in the Material and Methods is plotted versus increasing concentrations of sodium orthovanadate. The cells were incubated for 30 minutes at 37°C. Each point represents the mean ± S.E.M. of triplicate conditions. Similar results were obtained in at least two separate experiments.

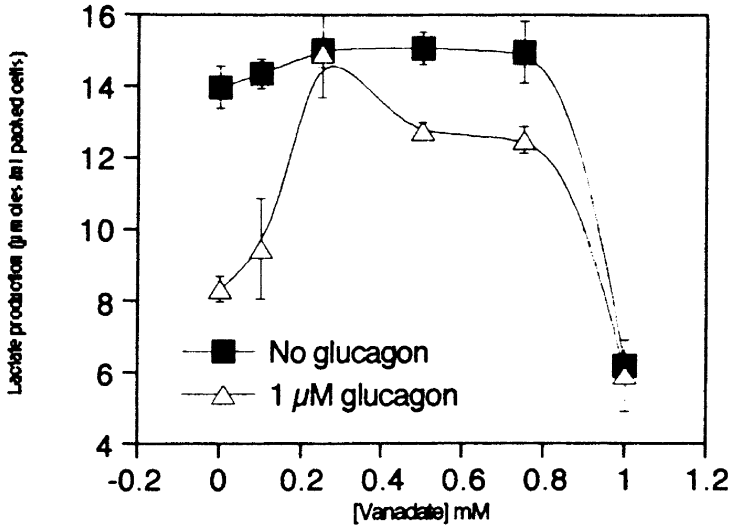
16
14
12
10
8
6
4
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
[Vanadate] mM
No glucagon
1 μM glucagon

TABLE 6

THE EFFECT OF VANADATE ON LACTATE FORMATION AND GLUCOSE PRODUCTION FROM 10 mM DIHYDROXYACETONE BY 1 μM GLUCAGON IN FRESH HEPATOCYTES FROM STARVED RATS

[Vanadate] μM	Glucose Production μmoles /ml packed cells		Lactate Production μmoles / ml packed cells	
	No Glucagon	1 μM Glucagon	No Glucagon	1 μM Glucagon
0	7.94±0.13 ^{y}	11.05±2.60^{x}	1.10±0.01^{a}	0.92±0.01a
1	8.13±0.48^{y}	10.99±0.39^{x}	1.12±0.04^{a}	1.06±0.02^{a}
10	7.78±0.44^{y}	10.32±0.46^{x}	1.64±0.04^{a}	1.46±0.09^{a}
50	6.71±0.43^{y}	9.91±1.27^{x}	2.03±0.04^{b}	1.98±0.08^{b}
100	6.11±0.74^{z}	8.64±0.89^{y}	2.07±0.06^{b}	2.06±0.06^{bc}
250	5.88±0.07^{z}	7.86±0.83^{y}	2.20±0.08 ^{b}	2.27±0.22^{bc}
500	4.08±0.23^{p}	7.28±0.18^{y}	2.24±0.05^{c}	2.29±0.02^{c}

The effect of different concentrations of vanadate on glucose and lactate formation from 10 mM dihydroxyacetone by isolated hepatocytes incubated at 37°C for 30 minutes, from starved rats was presented as means ±S.E.M. of triplicate conditions, in at least two separate experiments. Numbers with the same letter are not significantly different. $p < 0.01$

The Effect of Vanadate on Pyruvate Kinase Activity

Although vanadate regulates carbohydrate metabolism, and lowers blood glucose levels in diabetic rats Gil *et al.*, (1988), and Meyerovitch *et al.*, (1987), the effect at the lower concentrations takes longer . Therefore isolated hepatocytes were cultured for up to 36 hours to determine the effect of the anion on carbohydrate metabolism in long term culture. However, due to the changes that occur over long term culture, measuring the production of metabolites as carried out in short term experiments would have been more complicated. For example, glycogen accumulates which may interfere in the interpretation of results of the analysis of other metabolites. Changes in enzyme levels are easy to measure, and controls are simple to obtain, therefore enzymatic studies were initiated to find out the effect of vanadate on carbohydrate metabolism in relation to pyruvate kinase, a key regulatory enzyme in glycolysis. This enzyme was also chosen because Montserat *et al.*, (1991) reported that levels of vanadate as low as 25 μM were capable of raising mRNA levels of pyruvate kinase. Experiments were set up to determine the effect of 10, 50, and 100 μM sodium orthovanadate, and 10 nM insulin in the presence of physiological (5.5 mM) and diabetic (25 mM) levels of glucose on pyruvate kinase activity in cells cultured for 36 hours (Table 7). Relatively high concentrations such as 1mM vanadate previously used in the metabolic studies were dropped because they were observed to have an adverse effect on cell viability over the extended time periods used in primary culture. Table 7 shows the results of these experiments. 10 μM vanadate significantly increased PK activity as did 50 μM and 100 μM concentrations of the anion.

TABLE 7

THE EFFECT OF VANADATE ON PYRUVATE KINASE ACTIVITY IN CULTURED RAT HEPATOCYTES

Condition	PK Activity units/mg/DNA	
	5.5 mM Glucose	25 mM Glucose
Control	6.3±1.6[a]	7.5±1.4[ab]
10 nM Insulin	9.2±0.9[b]	8.5±0.6[ab]
10 μM Vanadate	25.1±7.0[d]	36.2±5.5[e]
50 μM Vanadate	12.2±1.3[bc]	18.0±3.1[c]
100 μM Vanadate	13.2±1.3[bc]	13.0±1.4[bc]

The effect of insulin, and different concentrations of vanadate on pyruvate kinase activity in isolated rat hepatocytes in primary culture for 36 hours. The table shows the mean ±S.E.M. of triplicate conditions, repeated in at least three experiments. Numbers with the same letter are not significantly different $p < 0.01$.

Vanadate Induction of Liver Pyruvate Kinase Gene Expression

Although Table 7 shows that vanadate raised pyruvate kinase activity in cultured liver hepatocytes, the data wasn't able to prove whether the increase in enzyme levels came from increase in actual protein activity, or a change in messenger levels due to a modulation of gene expression. To directly assess gene expression, gene constructs containing a 1000 base pair 5'-region of the pyruvate kinase promoter at the 5' end of the firefly luciferase gene were transfected into isolated hepatocytes in the presence of different concentrations of vanadate, insulin, and glucagon. Due to the fact that the transfection system is very sensitive to DNA concentration, this parameter had to be optimized by varying the amount of plasmid DNA put on each plate (Table 8), to ascertain the concentration that produced the most light (Relative Light Units, RLU). The amount of Lipofectin was kept constant at 20 μl per plate, since smaller quantities produced no transfection at all (data not shown). A linearity test was also carried out to determine if light units produced fell within the proportional range of the luciferase assay (Figures 3 and 4). The cells were incubated for 24 hours after transfection. Tables 9 and 10 show that insulin, vanadate, and glucagon increased gene expression of the pyruvate kinase promoter region under investigation. Table 9 shows that 10 μM vanadate had the greatest effect, while 10^{-6} M insulin was the concentration of the hormone that produced the most significant increase in gene expression. Lower concentrations of vanadate were still capable of raising PK gene expression levels (Table 10), with the response occurring at concentrations as low as 0.25 μM, in the presence of high (25 mM) glucose.

A glucose dose curve was set up to analyze whether the effects of vanadate and glucose were additive or synergistic (Table 11). From the results vanadate and glucose probably work in a synergistic manner. In all experiments two sets of controls were included. A blank, the promoter-less plasmid which never produced a relative light unit value of more than 11, and the positive control SV40. This plasmid indicated whether the transfection worked each time.

TABLE 8

OPTIMIZATION OF THE DNA CONCENTRATION REQUIRED FOR TRANSFECTION

DNA (μg)	RLU 24h post transfection incubation
0.00	7±0[a]
0.50	139±4.0[b]
0.75	238±25[c]
1.0	668±108[d]
2.0	232±18[c]
3.0	114±9[b]
4 .0	9±0[a]

Expression of pyruvate kinase gene in isolated hepatocytes incubated for 24 hours after transfection in 25 mM glucose and 10^{-6} M insulin. DNA amounts were varied to find the optimum amount for maximum light production. The data is presented as means ± S.E.M. n=3. Numbers with the same letter are not significantly different. $p < 0.01$.

Figure 3. Linearity of Luciferase Assay using Liver Cell Extracts.

Light emission (RLU) in the standard luciferase assay was determined using increasing amounts of extract (0, 10, 20, and 40 ul) from liver cells transfected with pGL2 plasmid containing liver pyruvate kinase promoter exposed to 25 mM glucose and 10^{-7} M insulin to test linearity of the assay. Details of the luciferase assay are provided in the Materials and Methods. Similar findings have been noted in several tests for linearity of the assay

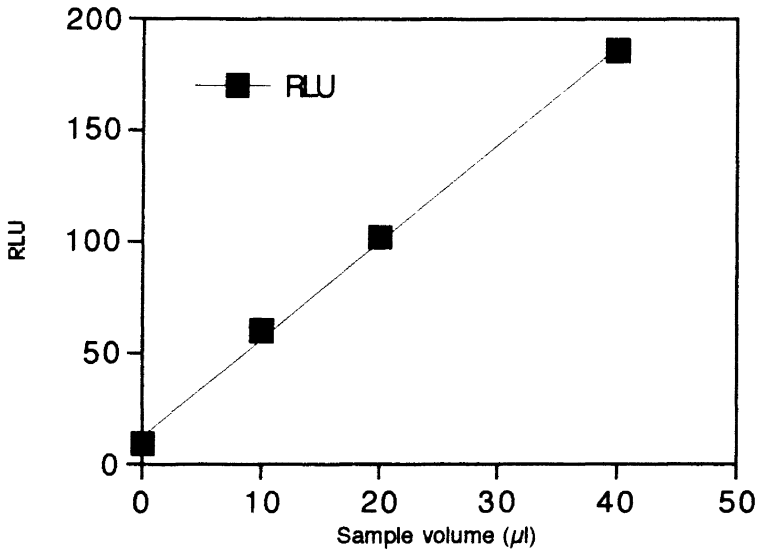
200
150
100
50
0
RLU
RLU
0
10
20
30
40
50
Sample volume (μl)

Figure 4 Luciferase Standard Curve.

40 µl of different concentrations of purified luciferase (Sigma) were assayed according to methods described in the Material and Methods section to determine whether light emission (RLU) was proportional to the amount of luciferase in the assay.

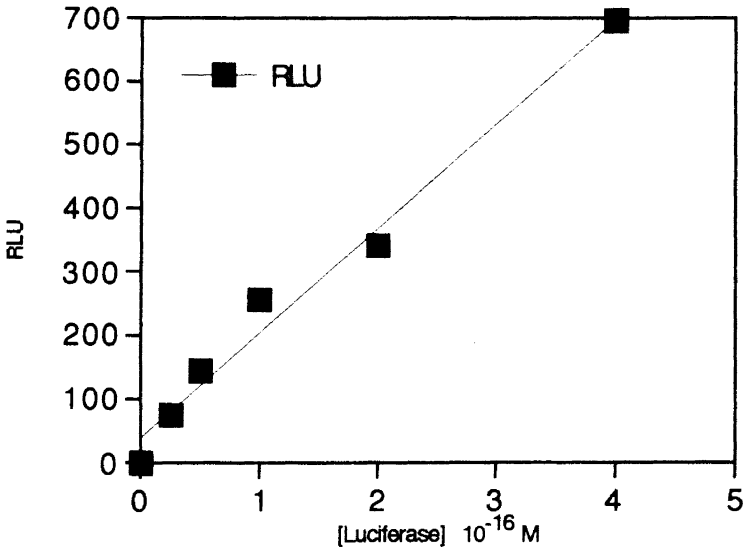
700
600
500
400
300
200
100
0
RLU
RLU
0
1
2
3
4
5
[Luciferase] 10^{-16} M

TABLE 9

MODULATION OF PYRUVATE KINASE GENE EXPRESSION IN ISOLATED RAT HEPATOCYTES BY VANADATE, INSULIN, AND GLUCAGON

Condition	RLU	
	10 mM Glucose	25 mM Glucose
Control	9 ± 1^{x}	24 ± 3^{a}
10^{-8}M Insulin	133 ± 11^{d}	136 ± 23^{d}
10^{-7}M Insulin	106 ± 7^{cd}	188 ± 52^{e}
10^{-6}M Insulin	115 ± 6^{cd}	244 ± 15^{ef}
10^{-10}M Glucagon	62 ± 5^{b}	72 ± 6^{bc}
$3x10^{-10}$M Glucagon	58 ± 4^{ab}	59 ± 6^{ab}
10^{-7}M Glucagon	126 ± 7^{cd}	111 ± 5^{cd}
1 μM Vanadate	90 ± 18^{bcd}	$342\pm25_{g}$
5 μM Vanadate	56 ± 2^{b}	224 ± 26^{e}
10 μM Vanadate	103 ± 3^{cd}	418 ± 5^{h}
SV40	not tested	247±10*
Promoter-less plasmid	not tested	9±0*

The effect of different concentrations of insulin, glucagon, glucose, and vanadate on the expression of pyruvate kinase gene in isolated hepatocytes 24 hours after transfection with plasmids containing a region of the pyruvate kinase promoter in front of firefly luciferase gene. Data represents mean ± S.E.M. of triplicate conditions. Similar responses were observed in at least two separate experiments. Numbers with the same letter are not significantly different. $p < 0.01$.

* In other experiments glucose and insulin have no effect on the SV40 plasmid (data not shown) and the promoter-less plasmid.

TABLE 10

VANADATE AND INSULIN REGULATE PYRUVATE KINASE GENE EXPRESSION IN RAT LIVER HEPATOCYTES

Addition	RLU	
	10 mM Glucose	25 mM Glucose
Control	137±28^{bc}	183±28^{c}
0.25 μM Vanadate	172±23^{c}	221±18^{d}
0.50 μM Vanadate	223±28^{d}	260±27^{e}
1.00 μM Vanadate	206±25^{cd}	276±25^{e}
2.00 μM Vanadate	192±15^{cd}	282±27^{e}
5.00 μM Vanadate	186±45^{c}	362±53^{g}
10.0 μM Vanadate	219±46^{cd}	218±26^{cd}
10^{-6}M Insulin	155±25^{bc}	435±26^{h}
10^{-7}M Insulin	135±22^{ab}	337±22^{f}
10^{-8}M Insulin	101±25^{ab}	264±21^{e}
10^{-9}M Insulin	73±6^{a}	187±35^{c}
10^{-10}M Insulin	142±45^{bc}	163±31^{bc}

The effect of low concentrations of vanadate, and insulin on pyruvate kinase gene expression 24 hours post-transfection in isolated rat hepatocytes exposed to two levels of glucose. Data represents mean ± S.E.M. of triplicate conditions. Numbers with the same letter are not significantly different. $p < 0.01$.

TABLE 11

THE EFFECT OF VANADATE, INSULIN, INCREASING GLUCOSE CONCENTRATIONS AND CERTAIN SUGARS ON PYRUVATE KINASE GENE EXPRESSION.

Addition	RLU				
	10 mM Glucose	20 mM Glucose	25 mM Glucose	30 mM Glucose	40 mM Glucose
Nothing	358±19^{c}	659±44^{e}	753±105^{ef}	722±51^{ef}	717±23^{ef}
10^{-6} M Insulin	487±27^{d}	871±55^{g}	805±84^{g}	1233±20^{i}	735±41^{ef}
1 μM Vanadate	730±44^{ef}	983±30^{h}	1139±54^{i}	1004±55^{gh}	1128±158^{ih}
5 μM Vanadate	662±70^{e}	1017±33^{gh}	686±55^{e}	770±97^{ef}	926±13^{g}
Dihydroxyacetone	295±34^{b}	*	*	*	*
Fructose	289±22^{b}	*	*	*	*
Glycerol	145±31^{a}	*	*	*	*

* Not determined.

The effect of increasing concentrations of glucose, on pyruvate kinase gene expression in the presence of vanadate, and insulin, and dihydroxyacetone, fructose, and glycerol. Data shows the mean of triplicate conditions ± S.E.M. Numbers with the same letter are not significantly different at $p < 0.01$

Discussion

Measurements of vanadate levels in the blood (Meyerovitch *et al.*, 1987) and liver (Gil *et al.*, 1990) of diabetic rats given low oral doses of sodium vanadate to lower blood sugar indicate that 15-30 μM vanadate is an effective concentration eliciting insulin-like actions on the intact rat. Although not directly measured, these studies indicate that vanadate is not concentrated to high levels in the liver. Tables 1 and 2 show that vanadate is not accumulated to high concentrations in isolated liver hepatocytes; rather the anion is taken up by cells until the intracellular concentration approximates that in the media. In rat liver hepatocytes the uptake of vanadate occurs rapidly, since cellular concentration reached extracellular range within 15 minutes. Longer term exposure in primary cells cultured in various concentrations of vanadate did not result in a concentration of the anion . These findings with liver contrasts with reports in yeast (Bode *et al.*, 1990) and erythrocytes (Nechay *et al.*, 1986). The accumulation of high amounts of vanadate in yeast cells appear to be due to vacuoles trapping the anion (Bode *et al.*, 1990). In erythrocytes vanadate appears to be reduced to vanadyl ion which is accumulated to high amounts. Therefore, liver may be distinct from these other specialized cells with regard to vanadate uptake and concentration. The importance of the present findings is that metabolic pathways influenced by vanadate in the liver result in lowered blood sugar, and therefore the liver must be sensitive to low concentrations of the ion if it is to reflect the situation in intact animals responding to vanadate.

Vanadate lowers blood glucose in diabetic rats (Meyerovitch *et al.*, 1987; Heyliger *et al.*, 1985; and Gil *et al.*, 1990). The anion may accomplish this by reducing glucose production in the liver either from glycogenolysis or gluconeogenesis. Results in this study show that vanadate inhibits glucose production in the liver from the aforementioned pathways. In hepatocytes from fed animals incubated without exogenous substrates vanadate lowers glucose production and counteracts the effect of glucagon (Table 5). High conentrations of vanadate such as 1mM are more effective and shifts the

glucagon dose response to the right. However the effects at high concentrations may be due to the toxic actions of the anion.Vanadate also inhibited glucose production from dihydroxyacetone in starved rats. In both types of experiments 50-100 μM vanadate were the lowest doses capable of producing a response. These levels are likely too high to account for the insulin-like actions of vanadate in diabetic rats.

Long term incubation of isolated hepatocytes with vanadate resulted in an increase in pyruvate kinase activity (Table 7). This indicates that the anion exerts effects on the cells that cause longer term adaptation in enzymes of carbohydrate metabolism. Table 7 shows that 10 μM vanadate significantly increased pyruvate kinase activity in cells cultured for 36 hours. Higher concentrations of the anion did not appear to work as well, possibly due to the toxic actions of higher concentrations of the anion. It appears that glucose enhances response to 10 μM vanadate. The concentration at which vanadate was most effective fell within the range capable of raising pyruvate kinase mRNA levels reported by Miralpeix *et al.*, (1991), and Montserat *et al.*, (1991), and with the observation that glucose enhances this response.

Having ascertained that vanadate increases pyruvate kinase activity in cultured hepatocytes, the next step was to assay for the regulation of promoter expression. Table 9 shows that vanadate was capable of inducing pyruvate kinase promoter expression in liver cells over a 24 hour incubation period. One, five, and ten μM vanadate significantly raised chimeric PK gene promoter expression in moderately high (10 mM), and very high (25 mM) glucose levels. The controls were a group of cells which were not exposed to insulin, vanadate, or glucose. There was a combined effect between glucose and vanadate because 25 mM glucose significantly increased gene expression under all conditions. . Concentrations of vanadate as low as 0.5 μM were found to significantly increase L-PK promoter expression. These effective concentrations are similar to those observed to raise PK mRNA levels (Miralpeix *et al.*, 1991). The lowest glucose concentration used in the present experiments was approximately 10 mM, because the incubation media contained

25% Waymouth which is rich in glucose (27 mM). Therefore, gene expression at physiological levels was not achieved in this study. Additional experiments utilizing media containing physiological, or no glucose are needed in the future to clearly define the role of the sugar. The other sugars dihydroxyacetone, glycerol, and fructose (all at a concentration of 15 mM) appeared ineffective. Thompson and Towle, (1991) reported that fructose increased pyruvate kinase promoter gene expression, but they used insulin their studies.

In the enzyme and gene expression experiments, insulin was used as a positive control. Although the hormone did not appear to raise pyruvate kinase activity in culture it did significantly increase pyruvate kinase chimeric gene expression especially in the presence of glucose (Tables 9 and 10). This dichotomy in insulin action was probably due to the sensitivity of the luciferase assay, and the rapid metabolism of insulin by the liver cells. Also in enzyme studies pyruvate kinase is initially present in the cells at significant concentrations. Therefore changes in enzyme activity are imposed on top of the initial level. In the gene expression experiments the cells do not normally produce luciferase, therefore any change is more easily measured. Insulin had a greater effect on gene expression in the presence of high levels of glucose, which is consistent with Thompson and Towle, (1991). These are effects that are more consistent with the insulin-like actions of vanadate and should be focused on in the future.

Glucagon did not give the results anticipated. Glucagon is reported to inhibit gene expression of pyruvate kinase in transfected hepatocytes (Thompson, and Towle, 1991), however Table 9 shows that the hormone induced expression. Thompson and Towle, (1991) used a chimeric construct containing at least 4000 bp of the 5' promoter region, while we used only a 1000 bp region. Additional chimeric constructs need to be developed to study glucagon actions and to identify the precise regions within the 1000 bp pyruvate kinase promoter region that are regulated by vanadate.

CHAPTER V

SUMMARY AND CONCLUSIONS

Vanadate has several insulin-like actions in isolated rat hepatocytes. The ability of the anion to inhibit glucose synthesis and to stimulate glycolytic formation of lactate in fresh hepatocytes require high concentrations of the anion and may be related to toxic actions of vanadate. However, in fresh hepatocytes, lower concentrations of vanadate appear to have some selective reversal in glucagon actions, suggesting a specific non toxic effect of the anion. Longer term incubation of hepatocytes from starved rats is accompanied by increased concentrations of the glycolytic enzyme pyruvate kinase, suggesting an action of the anion on gene expression. These responses were observed with vanadate concentrations as low as 0.5 -10 μM which corresponds to circulating levels of the anion capable of lowering blood glucose concentrations in diabetic rats. This phenomenon could be due to the effect of vanadate increasing the rate of transcription of pyruvate kinase, which stimulates glycolysis. Further studies need to be carried out to determine the exact location of the pyruvate kinase gene promoter that is modulated by vanadate.

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